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1. Donnelly, J.J., Ulmer, J.B., Liu, M.A. Minireview: DNA vaccines. Life Sciences vol. 6 no. 3, pp 163-172 (1997)

2. Ulmer, J.B., Sadoff, J.C., and Liu, M.A. DNA vaccines. Current Opinion in Immunology vol. 8, no. 4, pp 531-536 (1996)

3. Donnelly, J.J., Ulmer, J.B., and Liu, M.A. Immunization with DNA. Journal of Immunological Methods vol. 176 no.2, pp 145-152 (1994)

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DNA vaccines

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Preclinical DNA vaccine development has continued apace during the past year, with the investigation of several new infectious and non-infectious disease targets as well as advances in our understanding of some of the basic immunologic mechanisms, such as effector cells, responsible for conferring protection. The coming year promises to be at least as exciting, as initial human clinical studies have begun.

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Abbreviations

APC	antigen-presenting cell
CEA	carcinoembryonic antigen
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
GM-CSF	granulocyte macrophage-colony stimulating factor
HA	hemagglutinin
HBsAg	hepatitis B surface antigen
IL	interleukin
L1	major capsid protein
NP	nucleoprotein
PCR	polymerase chain reaction

Introduction

DNA vaccines are a recent addition to the armamentarium of potential vaccine technologies and offer promise for the improvement of existing vaccines. Furthermore, targets against which it has been difficult to make vaccines using existing methodology may become feasible if DNA vaccine technology is used instead. DNA vaccines consist of plasmid DNA expression vectors that, when administered to an animal, result in expression of an antigen *in situ* leading to the induction of antigen-specific immunity. The first demonstration of the protective efficacy of a DNA vaccine in an animal model was reported only three years ago [1]. Since then, studies have shown immunogenicity or protective efficacy of DNA vaccines in preclinical (animal) models for a variety of disease targets, and early human clinical studies have been initiated. Clearly, the validation of DNA vaccines awaits the results of such clinical studies and requires continuous evaluation of safety. DNA vaccines, however, offer a number of attractive attributes: their simplicity; the apparent robustness of the technology (a number of effective constructs have been made with off-the-shelf reagents); the breadth of their efficacy and applicability to various pathogens and diseases; and, unlike certain conventional vaccines, their ability to induce vigorous cellular immune responses. This article will review recent publications that expand the potential applications of DNA vaccines or that explore the immunologic mechanisms of protection.

Preclinical efficacy of DNA vaccines

Influenza

The initial demonstration of DNA vaccine efficacy in an animal model was accomplished using the influenza virus [1], which continues to provide a useful system with which to characterize immune responses to DNA vaccines. Influenza is one of the infectious disease targets for a DNA vaccine currently being investigated in human clinical trials. One rationale for efforts to develop an influenza DNA vaccine, despite the availablity of widely utilized existing influenza vaccines, is that current vaccines are effective only in a strain-specific manner. Mutations in circulating influenza virus strains mean that frequent re-evaluation and reformulation of the vaccine is necessary. DNA vaccines offer the advantage of stimulating the generation of cytotoxic T lymphocytes (CTLs) against epitopes from a conserved protein of the virus, such as nucleoprotein (NP), thereby providing cross-strain protection in a mouse model [1]. A study by Donnelly *et al.* [2•] further investigated the immunogenicity and protective efficacy of influenza DNA vaccines and compared them to the licensed conventional vaccines in nonhuman primates and in ferrets (which are considered to be the model of choice for influenza challenge studies because of their suseptibility to human clinical isolates of influenza virus). A combination DNA vaccine containing plasmids encoding both internal and coat proteins of the virus was prepared in a mix intended to mimic the proteins present in the whole virus vaccine. Vaccination of nonhuman primates generated titers of hemagglutination inhibiting antibodies (used as surrogates for neutralizing antibodies) as high as or higher than those generated by the full human doses of whole inactivated and split inactivated virus vaccines, respectively. Ferrets immunized with the DNA vaccine cocktail shed less virus in nasal washings after challenge with a drifted (i.e. antigenically different) strain of virus than did control animals immunized with the analogous commercially licensed whole inactivated virus vaccine. Furthermore, the level of protection induced by the DNA cocktail was statistically indistinguishable from that observed in homologous positive controls; that is, animals immunized with DNA encoding the hemagglutinin (HA) surface glycoprotein of the challenge virus (i.e. homologous virus). This study was significant for several reasons: it was a comparison of a DNA vaccine with a widely utilized licensed clinical vaccine; it was a demonstration of immunogenicity at low doses of DNA in nonhuman primates (10 μg of relevant construct per

dose, given twice; a regimen of two immunizations is customary for administration of the licensed vaccine in naive individuals); it was a demonstration of the efficacy of a cocktail DNA vaccine consisting of a mixture of plasmids encoding several antigens of the same virus; and it was a demonstration of efficacy against a naturally arising strain of virus that was antigenically distinct from the vaccination strain. The inability of the current vaccines to provide protection against such antigenically distinct viruses is one of their chief limitations which may thus be overcome by DNA vaccines.

Malaria

Another model in which a combination DNA vaccine was recently employed is malaria. It had been shown previously that DNA encoding the circumsporozoite protein of *Plasmodium yoelii* could induce CD8+ CTLs and confer protective immunity in mice [3]. The breadth of this protection was recently shown to be genetically restricted, however, although the inclusion of a DNA construct encoding another antigen (PyHEP17) circumvented this limitation by providing epitopes for another haplotype [4••]. Furthermore, Doolan *et al* [4••] demonstrated that interferon-γ and nitric oxide play key roles in protection. These results suggest that, in addition to induction of specific antibodies and CTLs, DNA vaccines may stimulate other less specific immune mechanisms to combat intracellular pathogens.

Other viral and bacterial disease models

Also important for bringing DNA vaccine technology closer to clinical application was the continued expansion of the breadth of preclinical infectious and noninfectious disease targets successfully tested in animal models. These included additional viral targets, as well as parasitic diseases, bacterial diseases, and cancer (against which the first clinical trials for DNA vaccines were initiated during 1995). Joining the viral disease targets for which immune responses and/or protection had already been demonstrated (such as influenza, bovine herpesvirus, hepatitis B, HIV and rabies) were hepatitis C virus [5,6], herpes simplex virus [7–10], papillomavirus [11], lymphocytic choriomeningitis virus [12–14] and flavivirus [15]. The list of parasitic diseases for which preclinical efficacy has been demonstrated was expanded from malaria [3] and leishmaniasis [16] to include schistosomiasis [17]. These results are important for several reasons. First, hepatitis C represents a disease for which no vaccine currently exists. Vaccine technology that can generate a CTL response might offer an important advantage over certain other types of vaccines because the viral core protein (best targeted by a CTL response rather than an antibody response) is relatively well conserved and because an antibody response alone might be of limited protective efficacy. Second, herpes simplex virus and papillomavirus are sexually transmitted pathogens with very high incidences of causing disease. Furthermore, their collective morbidity and mortality are not simply limited to primary viral infections per se, but include recurrent disease and, importantly, the potential for progression to carcinoma, in the case of papillomavirus. Third, the demonstration of protection in a rabbit model of papillomavirus [11] was significant from the standpoint of the immunobiology of DNA vaccines as well. In that report the DNA vaccine encoded the major capsid protein, L1, against which neutralizing antibodies are directed. When L1 is synthesized in an infected cell the protein is directed to the nucleus where nascent virus is assembled; epitopes against which neutralizing antibodies are directed are conformational in nature. Thus, it was somewhat surprising that a DNA vaccine encoding only L1 was capable of generating a protective antibody response in rabbits, as measured by prevention of condyloma development upon subsequent challenge with rabbit papillomavirus. These results indicate that L1 can attain a native conformation and be transported to a cellular location amenable to the generation of neutralizing antibodies in the absence of other papillomavirus proteins.

It is possible that bacterial diseases were not the initial target for DNA vaccines because a number of protective bacterial antigens are not proteinaceous (e.g. polysaccharides) and because less is known about the protective antigens of bacteria compared with viruses (i.e. those antigens that induce a protective immune response). For at least some bacterial targets, however, not only are proteins key antigens, but the types of cellular responses induced by DNA vaccines may be important elements of an effective vaccine. An example of such a bacterial target is *Mycobacterium tuberculosis*; the cellular responses against its proteins appear to play key roles in protective immunity. Recent work [18,19] has shown that tuberculosis DNA vaccines induce CTL and helper T cell responses of the Th1-like phenotype, as measured *in vitro* upon restimulation of spleen cells with antigen. Such responses have been reported with DNA encoding antigen 85 [18] and hsp65 [19]. Furthermore, protective efficacy conferred by DNA vaccines encoding these single *M. tuberculosis* antigens was comparable to that induced by the clinical Bacille Calmette-Guerin (BCG) vaccine which consists of a whole live organism whose efficacy is quite variable and which can cause disease in immunocompromised individuals.

Toward an understanding of immunologic mechanisms

Tolerance

From an immunologic standpoint, perhaps the most striking recent observations were obtained with hepatitis B DNA vaccines [20•,21•]. Mice transgenic for the surface antigen of hepatitis B (HBsAg) are tolerized by virtue of ontogenetic expression of the antigen. These mice express HBsAg in their hepatocytes and do not develop antibodies or CTLs against the expressed antigen, either spontaneously or after immunization with recombinant HBsAg. When they were immunized with a

plasmid encoding HBsAg, however, they developed both antibodies and CTLs with a concomitant elimination of antigen expression (Davis *et al.*, unpublished data). These results suggest that immunologic tolerance to the antigen in these transgenic animals was broken by this vaccination regimen. Interestingly, the expression of the transgene ceased without any evidence of immune-mediated hepatic damage, suggesting that elimination of HBsAg expression was mediated at a transcriptional or translational level rather than by destruction of antigen-expressing cells.

Increased breadth of immunogenicity

Other studies with HBsAg DNA have shown induction of immune responses in strains of mice that had previously shown little or no responsiveness to the antigen given as proteins. Mice of the H-2^b haplotype (C57BL/6) are considered to be nonresponsive to HBsAg at the CTL level because they do not generate MHC class I-restricted CTLs following immunization with either recombinant HBsAg or recombinant vaccinia virus expressing HBsAg. Vaccination with HBsAg DNA, however, generated both H-$2K^b$- and H-$2D^b$-restricted CTL responses to HBsAg epitopes [20•]. In mice with low responsiveness to HBsAg at the antibody level, HBsAg DNA was shown to induce immune responses following a single immunization, whereas use of the recombinant HBsAg required two immunizations before immune responses were seen [21•]. While these studies may not indicate that haplotype restriction was broken, they do indicate that antigen processing and/or presentation with DNA vaccines differs quantitatively and/or qualitatively from that found in other modes of vaccination.

Induction of CTLs

Until recently, CTL responses have been induced with DNA vaccines encoding full-size antigens: that is, entire proteins. In many cases this would be desirable to allow antigen processing and determinant selection in an outbred population. In some instances, such as for antigenically variable epitopes, however, it may be advantageous to direct the responses to specific epitopes of a protein. Such epitope-targeted immune responses have been induced using DNA vaccines containing minigenes encoding single CTL epitopes from mutant p53 and HIV gp120 [22]. In these cases it was found that enhanced CTL responses were attained when the peptides were preceded by an endoplasmic reticulum targeting signal sequence. This minigene approach should prove to be useful for induction of epitope-specific responses, especially for subdominant epitopes that may not induce responses in the presence of other more dominant epitopes.

One of the more intriguing unexplained issues regarding DNA vaccines is the nature of induction of CTL responses. The two more common means of administering DNA vaccines, intramuscular injection and particle bombardment, both induce MHC class I restricted CTLs. By the particle bombardment method, cells of the dermis and epidermis are transfected by the direct penetration of the DNA-coated gold beads. Since antigen presenting cells (APCs), such as Langerhans cells, are found in these layers of the skin; CTLs may be induced by direct transfection of these 'professional' APCs. In contrast, intramuscular injection results in the uptake of extracellular DNA by, and antigen expression in, muscle cells (and possibly in other cells, although this has not been documented). The importance of antigen expression by muscle cells in the induction of CTLs is not yet known. Several lines of evidence suggest that muscle cells may play a role. Firstly, while various routes of NP DNA administration can lead to the induction of CTLs, only intramuscular injection conferred substantial CTL-mediated protection from a cross-strain, lethal challenge with influenza virus [23]. Secondly, polymerase chain reaction (PCR) analyses of DNA from several tissues after intramuscular injection indicated that most, if not all, plasmid DNA was localized to the muscle [24•]. Therefore, while it is possible that intramuscular injection of DNA results in the transfection of nonmuscle cells, there is no evidence so far to support this possibility. Thirdly, transfection of nonmuscle cells is not required for the induction of MHC class I restricted CTLs and protective immunity, as demonstrated following transplantation of NP-expressing myoblasts into syngeneic mice [25]. Finally, the induction of CTLs after transplantation was mediated, at least in part, by transfer of antigen from the transplanted muscle cells to APCs of the recipient mice [25]. Further studies are required to delineate the cells responsible for CTL induction; however, taken together, these results suggest that intramuscular injection of DNA results in expression of antigen by muscle cells, but that the induction of CTLs is mediated by nonmuscle APCs, possibly by transfer of antigen from muscle cells to APCs.

Modulation of immune responses

In preclinical studies administration of DNA vaccines is an effective means of inducing antibodies and CTLs. The efficacy of DNA vaccines could be enhanced or modulated through the use of formulations that increase DNA stability or distribution in the muscle, the coexpression of immune molecules that affect the processing of antigens, or through the use of adjuvants that affect the immune responses that are mounted against the expressed antigen. With respect to adjuvants, in recent studies DNA vectors expressing cytokines have been shown to be biologically active *in situ* [26] and to affect immune responses against coexpressed antigens [27,28]. In the latter studies, granulocyte macrophage-colony stimulating factor (GM-CSF) was shown to have a stimulatory effect on both humoral and cellular immune responses to rabies virus glycoprotein [27] and carcinoembryonic antigen (CEA) [28]. Recombinant interleukins (ILs) -7 and -10 have also been used to increase the effectiveness of DNA vaccines in a tumor challenge model [29]. Others have used DNA encoding costimulatory molecules B-7.1 and B-7.2 in an effort to enhance or modulate immune responses

mounted against a coexpressed antigen by potentially providing an additional means of T cell stimulation. Using this approach, increased antibody responses against *M. tuberculosis* hsp65 were seen in a combination DNA vaccine containing B-7.2 DNA [19] and enhanced antibody and antitumor responses were induced by coinjection of DNA plasmids encoding CEA and B-7.1 [28].

Another potentially effective and simple way of enhancing immune responses to DNA vaccines is via an adjuvant effect of the DNA itself. Work over the past five years has shown that certain sequences can induce cytokine secretion and lymphocyte activation [30,31,32•]. Certain CpG motifs in bacterial DNA are particularly stimulatory, whereas similar DNA from other species are not; this is probably due in part to the methylation state of the DNA [32•]. Other CpG motifs can inhibit lymphocyte stimulation [31]. These observations suggest that manipulation of DNA vaccines to contain or avoid these motifs may affect the immunogenicity of antigens expressed by the vector. Preliminary observations have indicated that coinjection of a DNA plasmid (not expressing a protein) with a protein antigen (NP or HBsAg) modulates the isotype profile of the antibodies generated against the antigen, with a shift from an IgG1 predominance after injection of protein alone to an IgG2a predominance after injection of protein and DNA (JB Ulmer, CM DeWitt, RR Deck, MJ Caulfield, MA Liu, unpublished data). The immunomodulatory effect of bacterial DNA was also recently reported in an autoimmune mouse model. The results revealed that New Zealand Black/New Zealand White (NZB/NZW) mice spontaneously develop pathogenic antibodies to DNA and renal disease leading to premature death. Vaccination of pre-autoimmune NZB/NZW mice with bacterial DNA, but not mammalian DNA, accelerated the onset of anti-DNA antibody formation [33]. Unexpectedly, though, this vaccination regimen protected the mice from disease and death [34•], despite the fact that the DNA antibodies induced closely resembled the pathogenic antibodies seen in untreated mice. In addition, vaccination of mice in the advanced stages of disease prolonged their survival. Together with the aforementioned adjuvant properties of bacterial DNA, these results indicate that the composition of a DNA vector may be an important consideration in designing a DNA vaccine.

DNA delivery

Another way in which DNA vaccines could be modified is by the use of a delivery system, such as liposomes or polymers that can compact DNA and enhance cellular uptake, or the inclusion of peptides or proteins that can facilitate intracellular targeting of DNA to the cytoplasm and nucleus. In addition, DNA vaccines may be targeted to specific tissues such as mucosal sites for the induction of mucosal immune responses. To this end, Sizemore *et al.* [35•] have prepared a bacterial vector capable of DNA delivery. The attenuated *Shigella* they developed contains an *asd* (aspartic/semialdehyde dehydrogenase) mutation that does not interfere with the ability of the organism to invade cells but which causes it to burst open inside the cell, thereby releasing expression plasmids into the cytoplasm. Recombinant *Shigella* containing plasmids expressing galactosidase under the control of the human cytomegalovirus (CMV) early promoter and enhancer are not themselves able to express galactosidase but can direct expression of galactosidase in cell cultures, in the guinea pig eye, and in the mouse lung. Mice immunized in this manner produced cellular immune responses and high levels of specific antibodies. HIV and malaria genes have also been expressed in this system. These findings open up the possibility for the relatively inexpensive oral delivery of functional DNA with the potential for manipulation of the local immune system as well as for production of systemic responses (D Sizemore, A Branstrom, J Sadoff, unpublished data).

Expression library immunization

The identification of the protective antigens of a pathogen is a laborious and sometimes problematic process. This is particularly true for protection that requires cellular immunity, since certain types of vaccines (e.g. subunit proteins or whole inactivated viruses) do not generally induce CTLs. Furthermore, testing specific antigens requires that they be available in a purified form. With DNA vaccines, CTLs are readily induced and one needs only to have the gene encoding the antigen. The process of vaccine antigen discovery may be simplified by a recent and exciting application of DNA vaccine technology. Barry *et al.* [36••] developed a method to test mixtures of DNA plasmids containing fragments of the genome of a pathogen for protective efficacy (termed expression library immunization, or ELI). In their example, vaccination with mixtures containing 3000 distinct plasmids from a *Mycoplasma pulmonis* DNA library were shown to confer protection in a mouse challenge model, indicating that at least one of the plasmids encoded a protective antigen. By successive fractionation and testing of these mixtures it may be possible to identify the protective plasmids, although to date such fractionation and identification has not been reported. One of the potential drawbacks of the technique in addition to the potential masking of epitopes by immune interference is that, because fragments of the genome are used, many of the plasmids will not encode a relevant protein. This problem can be overcome by cloning open reading frames into the expression library and, with the burgeoning field of genomics, this sequence information is rapidly becoming available for many pathogens. Such approaches may greatly facilitate the identification of vaccine antigens.

Conclusions

The use of DNA vaccination has grown substantially in the three years since it was first demonstrated that DNA could confer protective immunity. Recent important advances have been made in several areas of DNA vaccines.

These include an expansion of the targets for DNA vaccine development, a greater understanding of some of the underlying mechanisms involved in the induction of immune responses, the beginnings of alternative DNA delivery vehicles that can target mucosal immune sites, the application of DNA vaccine technology to the discovery of protective antigens and the commencement of human clinical trials. Arguably the most significant of these is the latter, since the eventual success of DNA vaccines will be predicated on their effectiveness in humans.

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- •• of outstanding interest

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